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Joyce TAYLOR-PAPADIMITRIOU, et al.

Application No. 09/658,621

Filed: September 8, 2000

For: MUC-1 DERIVED PEPTIDES

Group Art Unit: Unassigned

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CLAIM FOR CONVENTION PRIORITY

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

The benefit of the filing date of the following prior foreign applications in the following foreign countries are hereby requested, and the right of priority provided in 35 U.S.C. § 119 is hereby claimed:

Great Britain Patent Application No. 9921242.5

Filed: September 8, 1999

European Patent Application No. 99 40 2237.4

Filed: September 10, 1999

In support of this claim, enclosed are certified copies of said prior foreign applications. Said prior foreign applications were referred to in the oath or declaration. Acknowledgment of receipt of the certified copies is requested.

Respectfully submitted,

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Date: December 6, 2000

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Bescheinigung | Certificate | Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

99402237.4

Der Präsident des Europäischen Patentamts;
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MUC-1 derived peptides

The present invention relates to antigenic polypeptides of the MUC-1 protein which are able to activate Cytotoxic T Lymphocytes (CTL) response and to nucleotide sequences encoding such polypeptides. Furthermore, the present invention relates to vectors comprising such nucleotide sequences, host cells comprising the same and their use in the production of the antigenic polypeptides. In addition, the present invention relates to compositions comprising the polypeptides, nucleotide sequences, vector or host cells of the present invention and to therapeutic and diagnostic uses of such compositions.

Generally speaking, there are two major types of immune response: the humoral response which is characterized by the production of antibodies by B lymphocytes, and the cell-mediated immune response. Antibodies are able to recognize antigens in their three dimensional form, either soluble or bound to an insoluble support such as a cell, while T cells recognize processed antigen fragments which are bound and presented by glycoproteins encoded by the major histocompatibility complex (MHC) notably MHC-I genes which are expressed at the cell surface of almost all vertebrate cells or MHC-II genes which are expressed on antigen presenting cells (APC).

A cell-mediated immune response usually necessitates the cooperation of helper T lymphocytes and effector cells. This cooperation takes place, in particular, as a result of interleukin-2 and/or various other cytokines which are secreted by helper T lymphocytes after their activation by antigenic fragments presented by APC in association with MHC-II. Cytotoxic T Lymphocytes (CTL) are activated, induced to proliferate and to exert their antigen-specific cytotoxic function upon exp sure to antigenic polypeptides complexed with

autologous MHC-I, co-stimulatory molecules on the surface of the APC and cytokines, often derived from helper T cells. T cell derived cytokines can also trigger and drive the proliferation and antigen processing capacity of APC as well as activation and induction of proliferation in other cells, including other T cells.

Said antigen presentation by MHC-I molecules has been characterized (see for example Groettrup et al, 1996, Immunology Today, 17, 429-435): a full-sized protein or glycoprotein antigen is digested into shorter antigenic polypeptides (of about 7 to 13 amino acids in length). Said polypeptides are associated with MHC-I molecules and β-2 microglobluin leading to a ternary complex which is further presented on the cell surface.

MHC-I specificity towards antigens can vary greatly depending on the considered MHC-I molecule (HLA-A, HLA-B, ...) and on the allele (HLA-A2, HLA-A3, HLA-A11, ...) since genes encoding the MHC molecules are greatly variable between individuals among a species (reviewed in George et al, 1995, Immunology Today, 16, 209-212).

Most tumor cells express antigens at their surface which differ either qualitatively or quantitatively from the antigens present at the surface of the corresponding normals cells. These antigens are specific when they are expressed only by tumor cells. When they are present on both normal and tumor cells, these antigens are said to be associated with the tumor; in this case, they are present either in larger amounts or in a different form in the tumor cells.

It is now well known that patients suffering from a cancer may develop an immune response to their tumour. This has been revealed, in particular, by demonstrating that the serum of some patients contain anti-tumor antigen antibodies, and that their serum was capable of inhibiting the growth of cancer cells in vitro. Nevertheless, inasmuch as spontaneous tumor regressions are extremely rare, it appears that the immune response observed in vitro remains ineffective in vivo.

Hellstrom et al. (1969, Adv.Cancer Res.12, 167-223) have shown that antigen-specific CTL can be effective mediators in a tumour-specific immune response. However, this

natural immune response is not always effective enough to limit tumor growth. Although an immune response may develop against a tumor, it is not known whether it is of real benefit to the patient. Seemingly uncontrolled tumour growth would suggest that a tumor eludes the body's mechanisms of immune surveillance. Tumour-derived molecules are considered to play a significant part in modifying or diverting the immune response in favour of the tumor rather in favour of the individual.

In the light of the complexity of the immune response against tumors and the modest state of current knowledge in this field, the use of an anticancer vaccine is not obvious. Animal studies have shown that immunization using living or killed cancer cells could lead to rejection of a subsequent tumor graft, however attempts at immunization using acellular products, for example administration of the complete antigenic protein, with polypeptide fragments of such protein DNA fragment encoding all or part of tumor-associated proteins, have generally been less successful.

Recently, Toes et al. (1997, Proc. Natl. Acad. Sci., 94, 14660-14665) have developed an alternative approach based on minimal antigenic polypeptide fragments selection which might be specifically recognized by the CTL. According to said method, the minimal antigenic fragments are expressed in the host cells where they can be associated with MHC-I molecules and then be presented on the cell surface, inducing a specific immune reaction. More specifically, it has been shown that intra-cellular expression of "minigens" encoding very short epitopes (from 7 to 13 amino acids in length) can induce a cellular immune response. Moreover, Whitton et al , (1993, J. of Virology, 67, 348-352) have proposed the use of a vector, called "string of beads" construct, which co-expresses several minigens and can induce a synergetic CTL immune response.

Another recent and important use for such polypeptides is in association with soluble complexes of MHC-I, β-2 microglobulin and a florescent or otherwise visually detectable reagent. These, so called "Tetramers" (*eg*, as described in Altman et al, 1996, Science, 274:94-96) can be used to identify by flow cytometry or histology, antigen specific CTL ex vivo.

MUC-1 is a glycosylated mucin polypeptide found on the apical surface of mucin-secreting epithelial cells in various tissues, including breast, lung, pancreas, stomach, ovaries, fallopian tubes, and intestine (Peat et al, 1992, Cancer Res.;52:1954-60 - Ho et al,

1993, Cancer Res.;53:641-51). Malignant transformation of breast, ovary, pancreas and probably other epithelial tissues, results in over expression of MUC-1 polypeptide in tumor cells (Hareuveni et al, 1990, Eur. Journ. Biochem.;189:475-86 ; Layton et al, 1990, Tumor Biol.;11:274-86). In addition, abnormal glycosylation of MUC-1 polypeptide in breast, and probably other MUC-1-expressing tumour cells results in the exposure of tumor-associated antigenic epitopes on the protein core of MUC-1 (Burchell et al, 1987, Cancer Res.;47:5476-82; Devine et al, 1990, J. Tumor Marker Oncol.;5:11-26; Xing et al, 1989, Immun.Cell Biol.:67:183-95) as well as on the glycosyl side chains (Samuel et al, 1990, Cancer Res.; 50:4801-8).

Monoclonal antibodies specific for these epitopes have been described which can identify more than 90% of breast and pancreatic tumors. Non major-histocompatibility-complex (MHC) restricted cytotoxic T cell responses to the MUC-1 tumor specific protein epitope by T cells from breast and pancreatic cancer patients have also been reported (Jerome et al, 1991, Cancer Res.;51:2908-16) in addition to MHC restricted, MUC-1-specific CTL (Reddish et al, 1995, Int J Cancer, 10:817-823). Moreover, proliferation of T cells to purified MUC-1 has been seen (Keydar et al, 1989, Proc.Natl. Acad. Sci. USA; 86:1362-6). These various observations suggest that MUC-1 may be an effective target antigen for active immunotherapy in breast, as well as other, cancers. Hareuveni et al (1991, Vaccine; 9:618-27) expressed the MUC-1 antigen in vaccinia virus and showed that rat immunized with VV-MUC-1 rejected MUC-1-bearing tumor cells at rate of 60-80 % (Hareuveni et al, 1990, Proc. Natl. Acad. Sci. USA; 87:9498-502).

The present invention concerns immuno-reactive polypeptides identified from the MUC-1 polypeptide sequence and their uses in cancer therapy and diagnosis. The invention could also be used to follow MUC-1 specific immune responses in patients during the course of disease and/or treatment. The invention also concerns nucleotide sequences encoding these polypeptides, vectors useful for transferring and expressing said nucleotide sequences into target cells, and uses of said nucleotide sequences in cancer gene therapy and diagnosis.

In accordance with the present invention polypeptides have been identified wherein said polypeptide comprises a polypeptide sequence of at most 20 consecutive amino-acids defined in SEQ ID NO: 1, wherein said polypeptide is different from SEQ ID NO : 2 and

is capable of binding with at least one MHC-I molecule.

"Capable of binding with" means that the considered polypeptide is capable to interact and to bind with MHC-I molecules. In a preferred embodiment of the invention, this binding results in cell surface presentation of these polypeptides by MHC class I molecules in order to elicit a specific immune response or for the detection of a specific immune response, eg, by Tetramer analysis (eg, as described in Altman et al, 1996, Science, 274:94-96).

According to a preferred embodiment, said polypeptide sequence is selected from the group consisting in SEQ ID NO: 3 to SEQ ID NO: 33. Data to explain why these sequences have been selected are shown in figures 1 to 7.

According to a first embodiment of the invention, said polypeptide presents at least one of the following properties :

(a) the polypeptide sequence is selected from the group consisting of SEQ ID NO:3 to SEQ ID NO:6, and said polypeptide binds the HLA A2 glycoprotein of MHC-I,

(b) the polypeptide sequence is selected from the group consisting of SEQ ID NO:7 to SEQ ID NO 15, and said polypeptide binds the HLA B7 glycoprotein of MHC-I,

(c) the polypeptide sequence is selected from the group consisting of SEQ ID NO: 16 to SEQ ID NO: 19, and said polypeptide binds the HLA A3 glycoprotein of MHC-I,

(d) the polypeptide sequence is selected from the group consisting of SEQ ID NO: 19 to SEQ ID NO: 21, and said polypeptide binds the HLA A11 glycoprotein of MHC-I,

(e) the polypeptide sequence is selected from the group consisting of SEQ ID NO: 22 to SEQ ID NO: 25, and said polypeptide binds the HLA A24 glycoprotein of MHC-I,

(f) the polypeptide sequence is selected from the group consisting of SEQ ID NO: 26 to SEQ ID NO: 29, and said polypeptide binds the HLA A1 glycoprotein of MHC-I,

(g) the polypeptide sequence is selected from the group consisting of SEQ ID NO: 30 to SEQ ID NO: 33, and said polypeptide binds the HLA B8 glycoprotein of MHC-I.

The invention is also related to polynucleotide comprising a nucleic acid sequence encoding at least one polypeptide of the present invention. According to a preferred embodiment, the nucleic acid sequence of the invention is selected from the group consisting of SEQ ID NO: 34 to SEQ ID NO: 64, and their complementary sequences.

The term "polynucleotide" as used in the scope of the present invention means a DNA and/or RNA fragment, single or double-stranded, linear or circular, natural or synthetic, modified or not (see US-A-5,525,711, US-A-4,711,955, US-A-5,792,608 or EP-A-0 302 175 for modification examples) defining a fragment or a portion of a nucleic acid, without size limitation. It may be, *inter alia*, a genomic DNA, a cDNA, an mRNA. "Polynucleotides" and "nucleic acids" are synonyms with regard to the present invention. The nucleic acid may be in the form of a linear polynucleotide, and preferably in the form of a plasmid. A wide range of plasmids is commercially available and well known by one skilled in the art. These available plasmids are easily modified by the molecular biology techniques (Sambrook et al, 1989, Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). Plasmids derived from pBR322 (Gibco BRL), pUC (Gibco BRL), pBluescript (Stratagene), pREP4, pCEP4 (Invitrogen) and also p Poly (Lathe et al., 1987, Gene 57, 193-201) are illustrative of these modifications. According to the invention, the nucleic acid can be a naked polynucleotide (Wolff et al., Science 247 (1990), 1465-1468) or is formulated with at least one compound such as a polypeptides, preferably viral polypeptides, oligonucleotides or cationic lipids, or cationic polymers which can participate in the uptake of the nucleic acid into the cells (see Ledley, Human Gene Therapy 6 (1995), 1129-1144 for a review) or a protic polar compound (examples are provided below in the present specification or in EP-A-0 890362). "Polynucleotide" also designates nucleic acid of viral origin (viral vector) which encodes at

least for the polypeptide f the invention. Such viral vector preferably derives from a virus selected among poxvirus (vaccine virus, MVA, canarypox....), adenovirus, retrovirus, herpes virus, alpha virus, foamy virus or adeno associated virus. Said viral vectors and their uses are widely disclosed in gene therapy literature.

Preferably, said nucleic acid includes at least one therapeutically useful gene sequence that can be transcribed and translated to generate a polypeptide of interest and the elements enabling its expression. The genetic information necessary for expression by a target cell comprises all the elements required for transcription of DNA into RNA and, if necessary, for translation of mRNA into a polypeptide. Transcriptional promoters suitable for use in various vertebrate systems are well known. For example, suitable promoters include viral promoters like RSV, MPSV, SV40, CMV or 7.5k, vaccinia promoter, inducible promoters, tissue specific promoters, synthetic promoters, etc or combination thereof. The nucleic acid can also include intron sequences, targeting sequences, transport sequences, sequences involved in replication or integration. Said sequences have been reported in the literature and can be readily obtained by those skilled in the art. The nucleic acid can also be modified in order to be stabilized with specific components as spermine.

The introduction or transfer process of an anionic substance of interest into a cell is by itself well known. "Introduction or transfer" means that the polynucleotide is transferred into the cell and is located, at the end of the process, inside said cell or within or on its membrane. It is also called "transfection" or "infection" depending of the nature of the vector.

The invention is therefore further directed to a vector, of viral or plasmid origin, comprising at least a nucleic acid sequence of the invention.

According to a preferred embodiment, the vector of the invention comprises one or more nucleotide sequences selected from the group consisting of:

- the sequences encoding a polypeptide as defined in (a) in addition to one or more of the sequences encoding a polypeptide as defined in (b), (c), (d), (e), (f) or (g),
- the sequences encoding a polypeptide as defined in (b) in addition to one or more of the sequences encoding a polypeptide as defined in (a), (c), (d), (e), (f) or (g),
- the sequences encoding a polypeptide as defined in (c) in addition to one or more of the sequences encoding a polypeptide as defined in (a), (b), (d), (e), (f) or (g),
- the sequences encoding a polypeptide as defined in (d) in addition to one or more of the sequences encoding a polypeptide as defined in (a), (b), (c), (e), (f) or (g),
- the sequences encoding a polypeptide as defined in (e) in addition to one or more of the sequences encoding a polypeptide as defined in (a), (b), (c), (d), (f) or (g),
- the sequences encoding a polypeptide as defined in (f) in addition to one or more of the sequences encoding a polypeptide as defined in (a), (b), (c), (d), (e) or (g),
- the sequences encoding a polypeptide as defined in (g) in addition to one or more of the sequences encoding a polypeptide as defined in (a), (b), (c), (d), (e) or (f).

The invention also encompasses a pharmaceutical composition which are particularly useful for the delivery of polynucleotides of the invention to cells or tissues of a subject in the scope of a gene therapeutic method , especially in case of cancer treatment. The term "gene therapy method" is preferably understood as a method for the introduction of a polynucleotide into cells either *in vivo* or by introduction into cells *in vitro* followed by re-implantation into a subject. "Gene therapy" in particular concerns the case where the polynucleotide is expressed in a target tissue, especially tissue comprising cell expressing MHC-I molecules.

Preferably, the pharmaceutical composition furthermore comprises a pharmaceutically acceptable carrier or diluent. The carrier or diluent are non toxic to recipients at the dosages and concentrations employed. Representative examples of carrier or diluent for injectable solutions include water, isotonic saline solutions which are preferably buffered at the physiological pH (such as phosphate buffered saline or Tris-buffered saline), mannitol, dextrose, glycerol and ethanol, as well as polypeptides or protein such as human serum albumin. This carrier or diluent is preferably isotonic, hypotonic or weakly hypertonic and has a relatively low ionic strength. Furthermore, it may contain any relevant solvents, aqueous or partly aqueous liquid carriers comprising sterile, pyrogen-free water, dispersion media, coatings, and equivalents. The pH of the pharmaceutical preparation is suitably adjusted and buffered.

The invention more particularly pertains to a pharmaceutical composition comprising at least one of the complexes described above and also incorporating at least one adjuvant capable of improving the transfection capacity of said complex. Adjuvants may be selected in the group consisting in a chloroquine, protic polar compounds such as propylene glycol, polyethylene glycol, glycerol, EtOH, 1-methyl L -2-pyrolidine or their derivatives, or aprotic polar compounds such as dimethylsulfoxide (DMSO), diethylsulfoxide, di-n-propylsulfoxide, dimethylsulfone, sulfolane, dimethylformamide, dimethylacetamide, tetramethylurea, acetonitrile or their derivatives.

In preferred embodiment, the polynucleotide which is contained in the pharmaceutical composition is a DNA. Other particular embodiments of the invention are pharmaceutical compositions wherein said polynucleotide is naked, associated with viral polypeptides or complexed with cationic components, more preferably with cationic lipids. In general, the concentration of polynucleotide in the pharmaceutical compositions is from about 0.1 µg/ml to about 20 mg/ml.

The pharmaceutical composition in accordance with the present invention can be administered into a vertebrate tissue. This administration may be made by intradermal, subdermal, intravenous, intramuscular, intranasal, intracerebral, intratracheal, intraarterial, intraperitoneal, intravesical, intrapleural, intracoronary or intratumoral injection, by means of a syringe or other devices. Transdermal administration is also contemplated, as are inhalation, aerosol routes, instillation or topical application.

According to the present invention, the pharmaceutical composition can be administered into target tissues of the vertebrate body including those of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, connective tissue, blood, etc. In a preferred embodiment, said composition will be administered into tumor.

Administration of such a composition to a patient allows to elicit an immune response based on the activation of cytotoxic lymphocytes by the polypeptides encoded by said nucleotide sequences. The composition of the invention is particularly suitable for the treatment of MUC-1-expressing cancers, such as breast cancer, ovary cancer, pancreas or lung cancer.

According to the invention, "cells" include prokaryote cells and eukaryote cells, yeast cells, plant cells, human or animal cells, in particular mammalian cells. In particular, cancer cells should be mentioned. The invention can be applied *in vivo* to the interstitial or luminal space of tissues in the lungs, the trachea, the skin, the muscles, the brain, the liver, the

heart, the spleen, the bone marrow, the thymus, the bladder, the lymphatic system, the blood, the pancreas, the stomach, the kidneys, the ovaries, the testicles, the rectum, the peripheral or central nervous system, the eyes, the lymphoid organs, the cartilage, the end thelium. In preferred embodiments, the cell will be a muscle cell, a haematopoietic system stem cell or an airways cell, a tracheal or pulmonary cell, or a tumor cell.

The present invention also encompasses a process for transferring a nucleic acid into cells wherein said process comprises contacting said cells with at least one polynucleotide according to the invention. This process may be applied by direct administration of said polynucleotide to cells of the animal *in vivo*, or by *in vitro* treatment of cells which were recovered from the animal and then re-introduced into the animal body (*ex vivo* process). In *in vitro* application, cells cultivated on an appropriate medium are placed in contact with a suspension consisting of polynucleotide of the invention. After an incubation time, the cells are washed and recovered. Introduction of the polynucleotide can be verified (eventually after lysis of the cells) by any appropriate method.

In the case of *in vivo* treatment according to the invention, in order to improve the transfection rate, the patient may undergo a macrophage depletion treatment prior to administration of the pharmaceutical preparations described above. Such a technique is described in the literature (refer particularly to Van Rooijen et al., 1997, TibTech, 15, 178-184).

The invention further concerns the use of a polypeptide or a polynucleotide , a vector or a host cell as defined above for the preparation of a pharmaceutical composition intended for diagnostic, curative, preventive or vaccination treatment of man or animals, and more specifically for the treatment of cancer.

The invention is also related to a diagnostic composition comprising at least one polypeptide as defined above. The use of a polypeptide of the invention in a diagnostic composition is illustrated by the following processes:

a process which enables the detection and eventually the quantification of an antibody directed against said polypeptide consists in (i) contacting with said polypeptide a biological sample susceptible to containing said antibody and (ii) detecting the formation of an immune complex between said antibody and said polypeptide.

a process which enables the detection and eventually the quantification of MUC-1-specific T lymphocytes according to the ELISPOT technique (Scheibenbogen et al, 1997, Clinical Cancer Research, 3, 221-226); Tetramer analysis (eg, as described in Altman et al, 1996, Science, 274:94-96) or other techniques which allow the identification of specific T cells by virtue of specificity of their T cell receptor for the polypeptides of this invention.

The methods, compositions, uses of the invention can be used for the treatment of all kinds of cancer the treatment and/or diagnostic of which being related to or dependent on the immune properties of the polypeptides of the invention. The compositions, and uses of the present invention may be desirably employed in humans, although animal treatment is also encompassed by the uses described herein.

These and other embodiments are disclosed or are obvious from and encompassed by the description and examples of the present invention. Further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on Internet, e.g. under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey

of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation. Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practised otherwise than as specifically described. Accordingly, those skilled in the art will recognize, or able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

The figures show:

Figures 1 to 7 show competition binding data from polypeptides whose sequences are from within the sequence of human MUC-1. Experiments were performed according to the method described in *van der Burg et al* (Hum. Immunol. 1995, 44:189-198). Polypeptides described as 'pp x' correspond to the SEQ ID NO. For example, pp 27 corresponds to SEQ ID NO 27. In some figures the competion binding curves of some negative (therefore not claimed) polypeptide sequences are shown to demonstrate the specificity of the competition binding assay.

Figures 8, 9 and 10 show ELISpot data from three experiments performed with PBMC, from patients immunized with VV-MUC-1-IL2, exposed to polypeptides from this invention. Spots per 10e6 PBMC indicate the number of CD8+ (CTL) T lymphocytes, per million PBMC, which are specific for that polypeptide. Black histograms represent the ELISpot responses of PBMC drawn from the patient 1 weeks after the injection of VV-MUC-1-IL2 (Figure 8), 4 weeks after injection (Figure 9) or 4 weeks after the second injection (Figure 10). The white bars correspond to the ELISpot response of patient PBMC taken before VV-MUC-1 administration (Figure 8) 5 months after injection (Figure 9) or before the second injection (Figure 10)

The examples illustrate the invention.

EXAMPLES

The following examples show:

1) The identification of MUC-1 polypeptides which bind specifically to molecules of the human Major Histocompatibility Complex I (MHC-I); and

2) The utility of these polypeptides in a functional bioassay, known as ELISpot (Enzyme Linked Immuno Spot) assay.

1) Competition Binding Assay

INTRODUCTION: Polypeptide fragments 8 - 13 amino acids long, of proteins produced within a nucleated, vertebrate cell are associated with newly formed cellular proteins of the MHC-I complex. The complex of MHC-I protein and polypeptide fragment are further associated with a protein known as Beta-2 microglobulin. This trimolecular complex is then transported to the cell surface, anchored to the cell membrane and exposed to the extracellular milieu. Thymus-derived, or T lymphocytes of the CD8 category have specific 'antigen' receptors on their cell surface, which recognise the MHC-I-Beta-2-microgobulin-peptide complex. Individual CD8+ T cells or clonal progeny of an individual T cell precursor express on their cell surface antigen receptors which recognise only one (or very few) such polypeptides within the context of the MHC-I-Beta-2-microglobulin-peptide complex. This is known as 'Antigen Specificity' of T lymphocytes. If the polypeptide is derived from a normal or 'self' protein,

T lymphocytes are not 'stimulated' due either to the deletion of self-specific T cells from the immune repertoire or due to negative regulation of self-specific immune responses. If, however, the polypeptide is from a pathogenic organism, such as a virus, then specific T cells are activated to proliferate and to become cytotoxic such that CD8 'cytotoxic effector cells' or CTL specifically recognise the infected cell and eliminate that cell in an effort to contain the pathogenic condition. Tumours can also produce 'tumour-specific' protein molecules or modifications of cellular proteins. In those cases, specific CD8 T cells can recognise a tumour cell as pathogenic and eliminate these cells by the same mechanism as is used to eliminate virus-infected cells. Often, the tumour-specific modification of a protein is merely quantitative in that a protein is over-produced in tumour cells. It has been shown that such proteins can also be recognised by specific Cytotoxic T Lymphocytes (CTL). For example see Disis *et al*, Cancer Research, 54: 1071-1076 (1994); or Barnd *et al*, Proc Natl Acad Sci USA, 86: 7159-7163 (1989).

It has been shown in numerous publications that the binding of said polypeptides to MHC-I molecules depends upon certain 'motifs' of amino acids at defined positions within the polypeptides. For example, the amino acids Leucine at position 2 and Valine at position 9 of a nine amino acid polypeptide will result in the binding of that polypeptide to HLA-A2. For a review, see Rammensee *et al*, Immunogenetics, 41: 178-228 (1995). The knowledge of the required amino acid positions had been acquired by the extraction of polypeptides from MHC-I molecules and sequencing them. In addition to the 'anchoring' residues there are various other 'preferred' flanking amino acids, such that a polypeptide can be given a 'rank' of likelihood that it will bind to a particular MHC-I molecule, depending on its sequence. Such ranking of predicted binding of polypeptides can be determined by one of several computer programs. In accordance with the invention, the program 'BIMAS' has been consulted (BioInformatics & Molecular Analysis Section) 'HLA POLYPEPTIDE Binding Predictions' (http://bimas.dcrt.nih.gov/molbio/hla_bind/) for predictions of which polypeptides from the human MUC-1 sequence are likely to bind to various HLA types. This, of course, is only a computer prediction and binding must be ascertained with a biochemical assay. Then, whether the polypeptides selected by the binding assay must be tested in a biological assay.

About 200 of the top ranking MUC-1 polypeptides, as predicted by the BIMAS program, predicted to bind to HLA-A1, A2, A3, A11, A24, B7 and B8 were produced (NeoSytem, Strasbourg, France) and were screened for HLA binding by a competitive binding assay. This assay is described in van der Burg *et al*, Human Immunology, 44:189-198 (1995). Briefly, EBV-transformed B lymphocyte

cell lines, of a known HLA-type, are exposed to a polypeptide known to bind to that HLA type. Binding of the polypeptide is determined by flow cytometry using a Fluorescence Activated Cell Sorter. Binding can b envisioned with this apparatus since the polypeptide known to bind is tagged with a flourescene molecule. Thus cells binding the polypeptide become fluorescent. Each polypeptide to be screened for binding is mixed together with the reference fluorescent polypeptide, which is at a constant concentration of 150 nM. The test polypeptide is added at various concentrations and the mixture exposed to the same cells. A test polypeptide is considered 'positive' if the binding of the reference polypeptide is inhibited by 50 % at 20 µg/ml or less of the test polypeptide. In figures 1 - 7 are shown data for the competitive binding of polypeptides deemed, by this assay, to be positive for binding to HLA-A2, B7, A3, A11, A24, A1, and B8 respectively. In each case, binding is compared to a negative control polypeptide, known not to bind to that HLA type and a positive control polypeptide, known to bind to that HLA type. For example, the positive control 'Flu' is a polypeptide with the sequence GILGFVFTL from the influenza virus matrix protein (Scheibenbogen et al. International Journal of Cancer, 71: 932-936 (1997).

MATERIALS:

EBV-B cell lines were derived by cultivating human Peripheral Blood Mononuclear Cells (PBMC) in filtered culture supernatant which had been used to grow cells from the marmoset line B-958 These cells produce the Epstein Barr Virus. PBMC are cultured for 2-3 days in the presence of 1µg/ml Cyclosporin A (to inhibit T cell reactivity to the virus) in the B-958 supernatant, then thereafter cultured in fresh culture medium. HLA-typed EBV-transformed human B cell lines were used for all tests HLA types are described in Table I.

Culture medium was Dulbecco's Modified Eagle's Medium (DMEM) + $5x10^{-5}$ M Beta-Mercaptoethanol (with the addition of 25 mM HEPES buffer for the step of polypeptide detachment and cell washing) plus either 2% or 10% Fetal bovine Serum. All tests were performed in 96 well, V-bottom microtitre plates (PS micro plate). β-2 microglobulin was purchased from Sigma. The buffer for the polypeptide detachment used was:

13.76 g citric acid (M MUC-1 210.14 g/L)
5.43 g Na_2HPO_4. $2H_2O$ (MMUC-1 177.9 g/L)
in 500 ml distilled H_2O

pH initially adjusted to pH 4.0, but pH then re-adjusted depending upon which HLA type is being tested (see Table I).

Dulbecco's Phosphate Buffered Saline (PBS) was purchased as a powder from Sigma. Reference polypeptides labelled with flourescene at the cysteine residue (Table I) were prepared according to Van de Burg et al., 1995, Hum. Immunol., 44, 189-198. Test polypeptides were purchased from NeoSystem, Strasbourg or prepared according to standard methods. Positive control polypeptides were purchased from NeoSystem (Strasbourg, France). Details of the polypeptides used are described in Table I.

Plastics, unless otherwise indicated, were purchased from Corning.

METHODS:

Cells were cultivated in Corning T175 flasks in 20 of culture medium (10% FBS). The night before the assay, cells were re-suspended and 10 ml of fresh medium added. The day of the test cells were resuspended, counted, pelleted by centrifugation and resuspended in 5 ml complete medium with 10% FBS. Distribute cells into a 6 well plate, 10^5 cells per well in 5 ml culture medium. Cells were then cultured for 4 hours at 37°, 5% CO_2. During that time polypeptides were prepared by dilution in 600 µg/ml in PBS. Two-fold serial dilutions from 600 - 4.68 (to have a final dilution of 100 to 0.78 µg/ml in the test plate) were first prepared in a separate 96 well 'polypeptide dilution' plate.

The test plate (96 well, V-bottom) was prepared as follows:

- negative control (no polypeptide): 50 µl PBS

- positive control (reference polypeptide only): 25 µl PBS + 25 µl Fl-reference polypeptide

- tests: 25 µl Fl-reference polypeptide at 150 nM (final) then 25 µl of test polypeptides (including positive and negative control polypeptides) at their various dilutions were added. Plates were then placed in a refrigerator in the dark.

After the 4 hour incubation of cells, the following were prepared, on ice:

- Two 15 ml conical bottom test tubes containing culture medium with 2% FBS.

- One 15 ml conical bottom test tube containing 10 ml culture medium with 2% FBS and including 1.5 µg/ml β-2 microglobulin

- One 15 ml conical bottom test tube containing 2 ml acid 'peptide detachment' buffer at the pH for the particular HLA type as indicated in Table I.

Cells in the 5 ml culture medium in the 6 well plate were resuspended and transferred to a 15 ml conical bottom test tube and then centrifuged for 5 minutes at 1500 rpm (500g). Resuspend the cells in PBS and centrifuge a second time (500g). Supernatant was removed and 2 ml detachment buffer while on ice. Cells were resuspended by gentle pipetting during the first 30 seconds of this 2 minute period. After 2 minutes, 14 ml culture medium with 2% FBS was added. Cells are mixed by inverting the tube twice, then centrifuged at 2000 rpm (800g) for 3 minutes at 4°C. Supernatant was removed and cells resuspended in 14 ml cold culture medium with 2% FBS and centrifugation repeated (3 minutes at 800g). Supernatant was removed and cells gently resuspended in 14 ml culture medium, 2 % FBS and 1.5 µg/ml β-2 microglobulin. One hundred µl cells from this suspension were added to each well of the 96 well plate, which already contained the polypeptides. The plate was wrapped in Saran Wrap and left 24 hours at 4°C. The next day, plates were centrifuged at 1000 rpm (200g), supernatant removed and cells resuspended in 100 µl PBS containing 0.1% Bovine Serum Albumin (BSA) and 0.02% sodium azide and cells pelleted by centrifugation at 200g. This step was repeated once more, then cells were resuspended in 1% paraformaldehyde and analysed for fluorescence by a FACScan (Becton Dickenson, Mountainview California).

The mean fluorescence intensity (MFI) of cells with the fluorescent reference polypeptide but with no competitor polypeptide (positive control) was taken as 0% inhibition. Similarly, the MFI of cells without the fluorescent reference polypeptide (negative control) was taken to be equal to 100 % inhibition. Percentage inhibition was calculated as:

$$\% \text{ inhibition} = \left(1 - \frac{(\text{MFI with test polypeptide}) - (\text{MFI negative control})}{(\text{MFI with test polypeptide}) - (\text{MFI positive control})}\right) \times 100$$

High Affinity Binding was taken to be 50 % inhibition at ≤ 10 µM polypeptide (~10µg/ml)

TABLE I: Reference and Positive C ntrol polypeptides used in the Competition Binding assay

Allele tested	elution pH	Reference Polypeptide			Positive Control Polypeptide	B-EBV Line	HLA Type
		sequence *(origin)*	Conc. pmol/µl	Final Conc. nM			
A1	pH 3.1	YLEPAC*AKY	183	150	CTELKLSDY *(Influenza NP 44-52)*	MAR	A01, A02, B08, B27, C01, C07
A2	pH 3.1	FLPSDC*FPSV *(HBV core 18-27)*	250	150	GILGFVFTL *(Influenza matrix 58-66)*	JY	A02, B07, C07
A3	pH 3	KVFPC*ALINK	28 et 20	150	QVPLRPMTYK *(HIV nef 73-82)*	FRE	A03, A24, B35, B08, C04, C07
A11	pH 3	KVFPC*ALINK	28 et 20	150		BVR	A11, B35, C04
A24	pH 3.1	RYLKC*QQLL *(HIV gp41 583-591)*	66 et 20	150	AYGLDFYIL *(melanoma p15 10-18)*	YT2	A24, B54, C01
B7	pH 3.1	APAPAPC*WPL *(human p53 84-93)*	29 et 20	150	RPPIFIRRL *(EBNA-3A 379-387)*	JY	A02, B07, C07
B8	pH 3.1	FLRGRAC*GI *(EBNA-3 339-347)*	20	150	YLKDQQLL *(HIV gp41 591-598)*	MAR	A01, A02, B08, B27, C01, C07

Where polypeptide origin is not indicated, HLA-A consensus sequences, as described in Sette *et al*, Molecular Immunolgy, 31: 813-822 (1994) were used. C* indicates that the flourescene molecule is coupled to a cysteine residue (as described in van der Burg *et al*, Human Immunology, 44: 189-198 (1995)).

Positive control polypeptides are published in Rammensee *et al*, 'MHC Ligands and polypeptide Motifs' Springer, New York (1995)

RESULTS:

The competition for binding to selected HLA types between serial dilutions of selected peptides and the reference peptides (as described in Table I) are shown in Figures 1-7. Binding of polypeptides, from the sequence of human MUC-1, to HLA -A2, B7,A3, A11, A24, A1 and B8 are shown in Figures 1,2,3,4,5,6, and 7 respectively. High affinity binding sequences were often, but not always, within the top 20 predicted binding polypeptide sequences as predicted by the BIMAS HLA Peptide Motif program (as described above).

2) ELISpot:

The ELISpot is a technique which allows the identification of antigen-specific (in this case, MUC-1-specific) T cell recognician by the detection of antigen induced production of cytokines (IFNγ, TNFα, IL-4, etc...) following an antigenic stimulation *in vitro*. More particularly, ELISpot allows the determination of the number of antigen specific T lymphocytes in a population of peripheral blood mononuclear cells (PBMC) (Scheibenbogen et al, 1997, Int. J. Cancer 71-1). In this case, the production of IFNγ produced by CD8+ T cells (CTL) in responses to polypeptides as presented by autologous HLA molecules were examined.

Briefly, in an ELISpot, the cytokines are captured between two specific antibodies. The first antibody, specific for human IFNγ, is adsorbed on a nitrocellulose membrane. Lymphocytes from human blood samples are added to the microtitre wells containing the attached antibody. Antigen, in the form of polypeptides, is also added to the wells. The principal is that polypeptides will attach to cell surface HLA molecules (together with β-2 microglobulin). Polypeptide specific T cells will recognize the complex of the polypeptide:HLA:β-2μglobulin. Upon recognition of antigen, the T cells become 'activated' to produce cytokines such as IFNγ. Secreted IFNγ is then captured by the antibody which is attached to the nitrocellulose. Cells are washed away leaving behind the areas of secreted IFNγ. These areas are revealed by the second antibody (coupled to biotin) and then by a streptavidin-alkaline phosphatase conjugate. The enzyme substrate hydrolysis by the enzyme leads to a spot appearance. Thus each spot represents the 'fingerprint' of a cytokine producing cell. The tests described below were performed using a commercially available kit (MABTECH, Nacka, Sweden)

MATERIALS

Peripheral blood mononuclear cells :

In Figures 8 and 9 patient PBMC (Peripheral Blood Mononuclear Cells) were obtained from breast cancer patients who had participated in a Phase I clinical trial carried out in the Institut Curie, Paris. In Figure 10, patient PBMC came from prostate cancer patients who had participated in a similar immunotherapy Phase I trial in Los Angeles, USA. In these trials, patients were immunized with a Vaccinia virus construct which expresses, upon infection, the both MUC-1 and IL2. The goal was to generate an immune response to MUC-1 which is an antigen over-expressed on both types of cancers. PBMC were isolated from peripheral blood by Hypaque-Ficol density centrifugation and resulting mononuclear cells frozen in aliquotes of 2 to $4x10^6$ cells in a 1 ml volume of culture medium containing 10% DMSO and stored in the vapour phase of liquid nitrogen until use.

TABLE II

Patient Identification Code	Cancer	HLA-Type
Pt#4 SOM	Advanced Breast	HLA-A 01/02; B 44/08
Pt#5 LEC	Advanced Breast	HLA-A 02/24; B 07/44
A002	Prostate	HLA-A01/26; B38/08

Tissue typing :

Breast cancer patient PBMC was HLA typed by serology and FCR at the Etablissement de Transfusion Sanguine, Strasbourg. Prostate cancer patients' PBMC were HLA typed by PCR at Transgene using the 'One Lamda' HLA typing kit (One Lamda, Canoga Park, CA, USA)

Polypeptides :

Polypeptides were produced at NeoSytem (Strasbourg, France)

ELISPOT :

The ELISPOT kit was purchased from and used according the instructions of MABTECH (Nacka, Sweden). The technique was carried out according to the manufacturer's instructions.

Briefly, PBMC were cultured in 96 well microtitre plates for 48 hours in the presence of test or control polypeptides at 5 µg/ml and recombinant IL-2 at 30 units/mL IFNγ spots were revealed with a second antibody reagent, also specific for Human Interferon gamma, according to the manufacturer's instructions.

RESULTS:

The results from three experiments are shown in Figures 8, 9 and 10. Patient PBMC were taken from liquid nitrogen and thawed the day before the ELISpot assay. The controls and the polypeptides (numbered according to their SEQ ID N°) were added as described above. Duplicate or triplicate wells containing 1- 2 x 10^5 PBMC were used. The number of spots was determined and is represented as the number of spots per 10^6 cells.

These data shown in figure 8 show that PBMC from patient #4 (who is HLA-A2) are able to respond to the polypeptide of SEQ ID NO: 4 in that PBMC from this patient are stimulated to produce IFNγ in response to the presence of this polypeptide but not in the presence of the negative control polypeptide or polypeptide SEQ ID N° 3. The response is seen after vaccination (black histograms), but not before (white histograms). In figure 9 is shown the results of an experiment in which PBMC from patient #5 (HLA-A2 and B7) are stimulated to produce IFNγ ELISpots upon exposure to polypeptide 4 (SEQ ID N° 4) and polypeptide 10 (SEQ ID N° 10) but not to the negative control or to polypeptides 3 or 7. No PBMC from prior to vaccination were available, but the patient's T cell response, as determined by *in vitro* CD4+ T cell proliferation assay, to a longer (24 aa) MUC-1 polypeptide was discernable only in the weeks following vaccination but was undetectable 5 months afterwards. The transient nature of the T cell responses is verified in Figure 9 in that only PBMC taken 28 days after vaccination (black histograms) were able to produce ELISpots over background, whereas PBMC taken 5 months after injection produced no ELISpot response to these peptide (white histograms).

These examples demonstrate the value of the invention in the diagnosis of a CD8+T cell immune response to MUC-1.

The invention could also be used in other diagnostic applications such as Tetramer analysis in which soluble MHC-I, beta-2-microglobulin and polypeptides of this invention are complexed together with a florescent reagent. The complex is then used to flourescently tag T cells with an antigen receptor specific for that polypeptide. The quantification of the specific T cells is accomplished with a florescence activated flow cytometer and can be done by one practised in the art.

The polypeptides of the invention could also be use in therapeutic or vaccine composition in order to prevent or treat MUC-1-expressing cancers. Polypeptides could be administered alone or complexed with MHC-I and beta-2-microglobulin to stimulate a MUC-1-specific CD8+ (CTL) T cell immune response. The invention could also be used as a DNA-based vector in which oligmucleotide sequences coding for the polypeptides of this invention, incorporated into viral or synthetic vector, are used to vaccinate a patient for the treatment or prevention of MUC-1-expressing cancers.

CLAIMS

1. A polypeptide consisting of or comprising an amino acid sequence of at most 20 consecutive amino-acids defined in SEQ ID NO 1, said polypeptide binding at least one MHC-I glycoprotein, with the proviso that said polypeptide is different from SEQ ID No: 2.

2. The polypeptide according to claim 1, wherein the amino acid sequence is SEQ ID NO 3 to SEQ ID NO 33.

3. The polypeptide according to claim 1 or 2, wherein the amino acid sequence is selected from the group consisting of:

   (a) SEQ ID NO 3 to SEQ ID NO 6, and said polypeptide binds the HLA A2 glycoproteins of MHC-I;

   (b) SEQ ID NO 7 to SEQ ID NO 15, and said polypeptide binds the HLA B7 glycoproteins of MHC-I;

   (c) SEQ ID NO: 16 to SEQ ID NO: 19, and said polypeptide binds the HLA A3 glycoprotein of MHC-I;

   (d) SEQ ID NO 19 to SEQ ID NO 21, and said polypeptide binds the HLA A11 glycoproteins of MHC-I;

   (e) SEQ ID NO 22 to SEQ ID NO 25, and said polypeptide binds the HLA A24 glycoproteins of MHC-I;

   (f) SEQ ID NO 26 to SEQ ID NO 29, and said polypeptide binds the HLA A1 glycoproteins of MHC-I; and

   (g) SEQ ID NO 30 to SEQ ID NO 33, and said polypeptide binds the HLA B8 glycoproteins of MHC-I.

4. A nucleotide sequence encoding a polypeptide according to any one of claims 1 to 3.

5. The nucleotide sequence according to claim 4, wherein said nucleotide sequence is selected in the group consisting of SEQ ID NO 34 to SEQ ID NO 64, and their complementary sequences.

6. The nucleotide sequence according to claim 4 or 5, wherein said nucleotide sequence contains elements allowing its expression in a host cell.

7. The nucleotide sequence according to claim 6, wherein said element for expression in a host cell is a promoter.

8. The nucleotide sequence according to any one of claims 4 to 7, wherein said nucleotide sequence is associated with one or more compounds selected from transfecting agents, stabilizing agents, or targeting agents.

9. Vector comprising at least one nucleotide sequence according to claims 10 to 15.

10. The vector according to claim 9 comprising at least two different nucleotide sequences encoding at least two polypeptides as defined in claim 3.

11. The vector according to claim 9 or 10, wherein said vector is a plasmid.

12. The vector according to claim 9 or 10, wherein said vector is a viral vector.

13. A host cell comprising at least a nucleotide sequence according to any one of claims 4 to 8 or at least a vector according to any one of claims 9 to 12.

14. The host cell according to claim 13, wherein said cell is a prokaryotic cell, yeast cell, or animal cell such as mammal cell.

15. A composition comprising at least a nucleotide sequence according to any one of claims 4 to 8, a vector according to any one of claims 9 to 12, or a host cell according to claim 13 or 14.

16. The composition according to claim 15, further comprising a pharmaceutical carrier.

17. Use of a nucleotide sequence according to any one of claims 4 to 8, a vector according to any one of claims 9 to 1, a host cell according to claim 13 or 14 or a composition according to claim 15 or 16 for the preparation of a medicament for effecting a CTL response in a subject.

18. A composition comprising at least one polypeptide according to any one of claims 1 to 3.

19. Use of at least one polypeptide according to any one of claims 1 to 3 for the preparation of a diagnostic or pharmaceutical composition.

ABSTRACT

Described are peptides and polypeptides derived from the MUC-1 polypeptide which are able to activate Cytotoxic T Lymphocyte (CTL) response, nucleotide sequences encoding such peptides and polypeptides and therapeutic uses thereof. Moreover, indications for selecting appropriate minimal antigenic MUC-1 polypeptides with reference to the HLA-type of the patient to be treated or tested are described.

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65                  70                  75                  80

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Pro Ala Ser Lys Ser Thr Pro Pro Ser Ile Pro Ser His His Ser Asp
225 230 235 240

Thr Pro Thr Thr Leu Ala Ser His Ser Thr Lys Thr Asp Ala Ser Ser
245 250 255

Thr His His Ser Thr Val Pro Pro Leu Thr Ser Ser Asn His Ser Thr
260 265 270

S r Pro Gln Leu Ser Thr Gly Val Ser Phe Phe Phe Leu Ser Phe His
275 280 285

Ile Ser Asn Leu Gln Phe Asn Ser Ser Leu Glu Asp Pro Ser Thr Asp
290 295 300

Tyr Tyr Gln Glu Leu Gln Arg Asp Ile Ser Glu Met Phe Leu Gln Ile
305 310 315 320

Tyr Lys Gln Gly Gly Phe Leu Gly Leu Ser Asn Ile Lys Phe Arg Pro
325 330 335

Gly Ser Val Val Val Gln Leu Thr Leu Ala Phe Arg Glu Gly Thr Ile
340 345 350

Asn Val His Asp Val Glu Thr Gln Phe Asn Gln Tyr Lys Thr Glu Ala
355 360 365

Ala Ser Arg Tyr Asn Leu Thr Ile Ser Asp Val Ser Val Ser His Val
370 375 380

Pro Phe Pro Phe Ser Ala Gln Ser Gly Ala Gly Val Pro Gly Trp Gly
385 390 395 400

Ile Ala Leu Leu Val Leu Val Cys Val Leu Val Ala Leu Ala Ile Val
405 410 415

Tyr Leu Ile Ala Leu Ala Val Cys Gln Cys Arg Arg Lys Asn Tyr Gly
420 425 430

Gln Leu Asp Ile Phe Pro Ala Arg Asp Thr Tyr His Pro Met Ser Glu
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Tyr Pro Thr Tyr His Thr His Gly Arg Tyr Val Pro Pro Ser Ser Thr
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Asp Arg Ser Pro Tyr Glu Lys Val Ser Ala Gly Asn Gly Gly Ser Ser
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Leu S r Tyr Thr Asn Pro Ala Val Ala Ala Thr Ser Ala Asn Leu
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HLA-A2:
MUC1 Polypeptide Competition
Binding Assay
Flu pp (pos control)
pp neg control
pp 6
pp 4
pp 5
pp 3
% inhibition
0
10
20
30
40
50
60
70
80
90
100
1
10
100
ug/ml Polypeptide (pp)

FIGURE 1

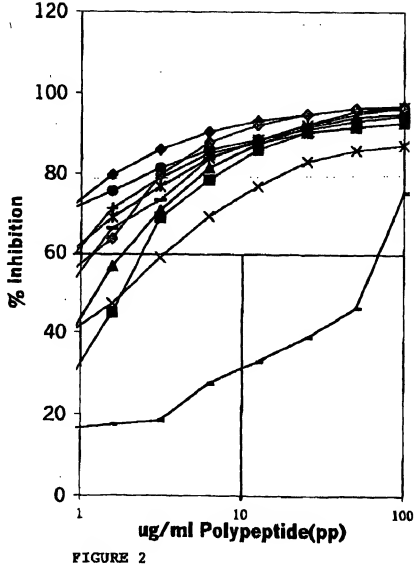
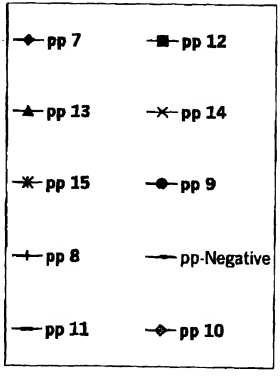


FIGURE 2

HLA-B7:
MUC1 Polypeptide
Competition Binding Assay



HLA-A3:
MUC1 Polypeptide
Competition Binding Assay
pp-pos control
pp 19
pp 20
pp 16
pp 17
pp 18
pp Negative
% inhibition
100
90
80
70
60
50
40
30
20
10
0
1
10
100
ug/ml Polypeptide (pp)

FIGURE 3

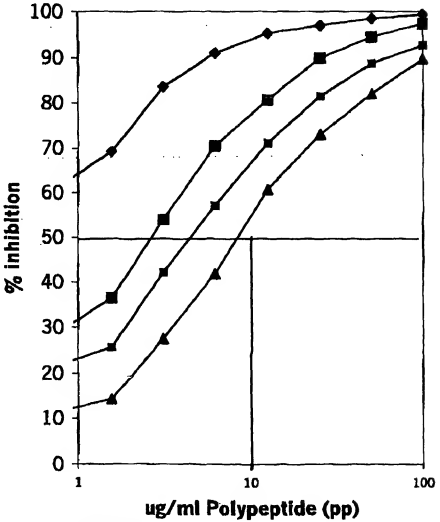
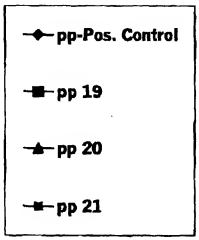


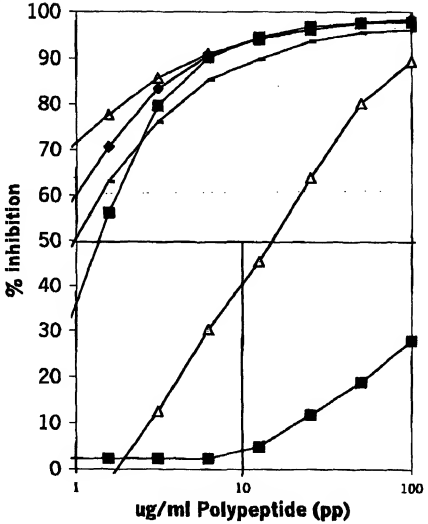
FIGURE 4

HLA-A11:
MUC1 Polypeptide
Competition Binding Assay:



HLA-A24 :
MUC1 Polypeptide
Competition Binding Assay
pp 22
pp 23
pp 25
pp-Pos, Cont.
pp-Negative
pp 24
% Inhibiti n
100
90
80
70
60
50
40
30
20
10
0
1
10
100
ug/ml Polyeptide (pp)

FIGURE 5



HLA-A1 :

MUC1 Polypeptide Competition Binding Assay:

- pp-Pos Cont
- pp 26
- pp 29
- pp 28
- pp 27
- pp-Negative

FIGURE 6

HLA-B8
MUC1 POLYPEPTIDE
COMPETITION BINDING ASSAY
pp - Pos. Cont.
pp 32
pp 30
pp 31
pp-Negative
pp 33
% Inhibition
100
90
80
70
60
50
40
30
20
10
0
1
10
100
ug/ml Polypeptide (pp)

FIGURE 7

Spots per 10e6 cells
0
10
20
30
40
Negative
Polypeptide 4
Polypeptide 3
Peptides

FIGURE 8

Spots per 10e6 cells
0
10
20
30
40
Negative
polypeptide 3
polypeptide 4
polypeptide 10
polypeptide 7
Polypeptides

FIGURE 9

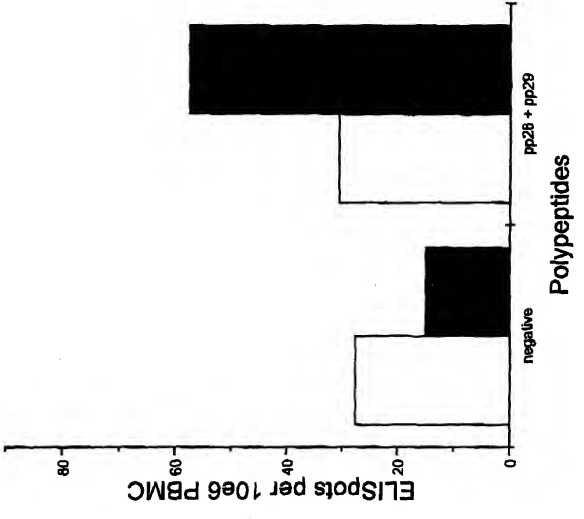

ELISpots per 10e6 PBMC
0
20
40
60
80
negative
pp28 + pp29
Polypeptides

FIGURE 10